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(54) **Title:** METHOD AND KIT FOR DETECTING A RISK FOR DIABETES OR A METABOLIC SYNDROME

(57) **Abstract:** The invention provides a method and kit for detecting or diagnosing a risk of or predisposition to diabetes or a metabolic syndrome in a subject, the method comprising the steps of providing a biological sample of the subject to be tested and detecting the presence or absence of a variant genotype of the human α_{2B}- adrenoceptor in the biological sample. The presence of the variant genotype indicates an increased risk of diabetes or a metabolic syndrome in said subject. The invention also relates to a method for the treatment of type 2 diabetes.

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Method and kit for detecting a risk for diabetes or a metabolic syndrome.

FIELD OF THE INVENTION

The invention provides a method and kit for detecting or diagnosing a risk of or predisposition to diabetes or a metabolic syndrome in a subject, the method comprising the steps of providing a biological sample of the subject to be tested and detecting the presence or absence of a variant genotype of the human α_{2B}-adrenoceptor in the biological sample. The invention also relates to a method for the treatment of diabetes.

BACKGROUND OF THE INVENTION

There are two sub-types of α-adrenoceptors: the α_1-adrenoceptors (α_1-ARs), on the effector organs (postsynaptic) and the α_2-adrenoceptors (α_2-ARs) on the nerve endings (presynaptic). The α_2-adrenoceptors mediate many of the physiological effects of epinephrine and norepinephrine including a reduction of release of norepinephrine. Three genetic subtypes of α_2-ARs are known in humans, denoted as α_{2A}-, α_{2B}- and α_{2C}-AR that are located on chromosomes 10, 2 and 4, respectively (Calzada and Artinano 2001). The tissue distributions and physiological and pharmacological functions of the receptor subtypes have been reviewed elsewhere (Docherty 1998, Calzada and Artinano 2001). Based on recent studies with gene-targeted mice, α_{2A}-ARs mediate most of the pharmacological actions ascribed to currently available α_2-AR agonists, including inhibition of neurotransmitter release, central hypotensive and bradycardic effects, sedation and anaesthesia, and analgesia (MacMillan et al 1998).

The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several different neurotransmitters are stored within the terminals of these nerves, the classical neurotransmitters, acetylcholine and norepinephrine, and neuropeptides, which exert local effects. Stimulation of the autonomic nerves affects islet hormone secretion.Thus, insulin secretion is stimulated by parasympathetic nerves or their neurotransmitters and inhibited by sympathetic nerves or their neurotransmitters. The islet autonomic nerves seem to be of physiological importance

allowing oscillations of islet hormone secretion and are also involved in the islet adaptation in disease states (Ahrén 2000).

Sympathetic nervous system (SNS) over-activity plays a key role in the development of the metabolic syndrome, which is characterised by the combination of high blood pressure, increased glucose production, decreased glucose utilisation, increase in triglycerides and VLDL, decrease in HDL and insulin resistance (Borchard 2001). Insulin resistance is defined as an impaired biologic response to exogenous or endogenous insulin and is a state in which the ability of insulin to suppress hepatic glucose output and stimulate the uptake and utilisation of glucose by muscle and adipose tissues is impaired (American Diabetes Association 1998).

The effects of the SNS on glucose and lipid metabolism are mediated by circulating catecholamines (epinephrine and norepinephrine) and the direct sympathetic innervation of the liver, adipose tissues and skeletal muscle. It has been generally recognised that increased sympathetic neural activity (SNA) produces catabolic effects on glucose and lipid metabolism (Nonogaki 2000) and inhibits insulin release from pancreatic beta-cells and glucose uptake into skeletal muscle cells, which are a major site of insulin-mediated glucose uptake (Borchard 2001) and a primary site of insulin resistance as measured by euglycaemic glucose clamp technique (Moan et al. 1996). Increased SNA causes vasoconstriction of the skeletal muscle arteries leading to impaired glucose tolerance and to insulin resistance.

In isolated adipocytes beta-adrenergic stimulation induces a rapid down-regulation of insulin receptors together with a decrease in insulin-mediated glucose transport. Insulin resistance leads to a breakdown of stored triglycerides in the adipose tissue and an increase in plasma free fatty acids. As a consequence, hepatic synthesis of triglycerides from free fatty acids and conversion of triglycerides to VLDL-cholesterol is enhanced. Catecholamines may further increase lipolysis in adipocytes which results in an elevated release of free fatty acids into the blood stream. Free fatty acids decrease glucose-stimulated insulin release from the pancreas, which further enhances glucose intolerance. Furthermore, catecholamines may inhibit lipoprotein lipase and thus increase VLDL, which is linked to a decrease in HDL (Borchard 2001). The end result

is a steady state of hyperinsulinemia. Support for this concept is based on these observations: a) a decreased skeletal muscle capillary density has been found in insulin-resistant states of hypertension, obesity, and type 2 diabetes; b) antihypertensive drugs that cause vasoconstriction worsen insulin resistance and those that cause vasodilation improve insulin sensitivity; and c) exercise training improves insulin sensitivity and increases skeletal muscle capillary density (Julius et al. 1991).

The role of SNS in insulin resistance and diabetes is further strengthened by studies in which blockade of parasympathetic activity has been shown to increase the prevalence and severity of diabetes in mice suggesting role of unopposed sympathetic activity (Kvist-Reimer et al. 2002) and sympathectomy abrogated the development of both hyperinsulinemia and hypertension in fructose hypertensive rats - a widely used model to study the inter-relationship between hyperinsulinemia, insulin resistance and high blood pressure (Verma et al. 1999).

In addition to the findings that increased SNA promotes insulin resistance and type 2 diabetes there is also experimental evidence that hyperinsulinemia may itself increase sympathetic neural outflow by activating anteroventral portion of the third ventricle in the central nervous system, a region implicated in arterial pressure regulation and sympathetic neural control. In rats and humans acute insulin elicits increased lumbar SNA along with elevated plasma norepinephrine and studies have firmly established in both humans and experimental animals that insulin administration produces marked increases in SNA directed largely to skeletal muscle tissues (Muntzel 1999). Insulin is also involved in the initiation of appropriate changes in sympathetic outflow within the central nervous system in response to diet. During fasting state, the small decline in the plasma glucose concentration and the larger decrease in the plasma insulin concentration result in diminished insulin-mediated uptake and metabolism of glucose to carbon dioxide by insulin-sensitive cells in the ventromedial hypothalamus. The decreased glucose metabolism stimulates the activity of an inhibitory pathway between the hypothalamus and the brain stem, suppressing tonically active sympathetic centres in the brain stem and decreasing central sympathetic activity. Conversely, in insulin-resistant states, the increase in plasma glucose and the greater increase in plasma insulin (hyperinsulinemia) stimulate insulin-mediated uptake and metabolism of glucose by the hypothalamic cells.

The increase in glucose metabolism diminishes the activity of the inhibitory pathway, disinhibiting tonically active brain-stem centres and increasing central sympathetic activity leading to hypertension by stimulating the heart, the vasculature, and the kidneys (Reaven et al. 1996).

Thus it can be concluded that increased SNA and hyperinsulinemia by a mutually dependent process results in insulin resistance and increased risk of type 2 diabetes and carries a high mortality and risk of cardiovascular diseases, kidney failure and infectious complications as measured by resistance and pulsatility indices (Takahashi et al. 1998; Valensi et al. 1997; Weinrauch et al. 1995; Weston et al. 1999) and warrants an early detection and treatment.

Both β-adrenoceptor antagonists (β-blockers) and diuretics have been observed to influence glucose balance unfavourably, elevating blood glucose and serum insulin levels. Drug-induced hyperglycemia is a growing concern. Several antihypertensive drugs have an adverse effect on glucose tolerance that may partially or completely negate the beneficial effects of reducing blood pressure as it relates to the incidence of coronary heart disease and its complications. Diuretics and beta-blockers have the greatest adverse effect on glucose intolerance (Houston 1986) and patients receiving beta–blocker treatment or diuretics may be at increased risk for developing hyperglycemia and subsequent diabetes mellitus (Luna et al. 2001; Bengtsson 1984; Gress et al. 2000; Bengtsson et al. 1984).

Metabolic studies of beta-blockers and diuretics aroused concern about the diabetogenic potential of these drugs. Subsequently, the results of prospective cohort studies and clinical trials suggested a causal link between the use of beta-blockers or diuretics and the subsequent development of type 2 diabetes (Gress et al. 2000; Bengtsson et al. 1984; Lithell 1998; Papaccio et al. 1987). The risk of diabetes was 28 percent greater among those who took beta-blockers than among those who took no medication, without regard to the presence or absence of hypertension, sociodemographic characteristics, health-related behaviour, family history with respect to diabetes, and a variety of coexisting conditions (Gress et al. 2000). Likewise, some observational studies have identified higher estimates of relative risk: for instance, in one study,

subjects who took beta-blockers had up to 6.1 times the risk of diabetes of those who did not (Samuelsson et al. 1994).

Thiazide diuretic–induced hyperglycemia occurs primarily through the reduction in total body potassium and the subsequent decreased insulin secretion (Luna et al. 2001; Greenberg 2000; Perez-Stable et al. 1983) by the beta cells, and reductions in extracellular fluid volume and cardiac output. This is compounded by increases in catecholamines from sympathetic nerve activity, which decrease peripheral glucose utilisation (Wilcox 1999). Potential mechanisms by which beta-blockers may contribute to the development of diabetes include weight gain, attenuation of the beta-receptor–mediated release of insulin from pancreatic beta cells, and decreased blood flow through the microcirculation in skeletal-muscle tissue, leading to decreased insulin sensitivity (Sowers et al. 2000).

SUMMARY OF THE INVENTION

The object of this invention is to provide a screening method to assess if an individual is at risk to develop diabetes or a metabolic syndrome based on the genotype of α_{2B}-adrenoceptor (ADRA2B) gene. The invention also provides a method for the treatment of diabetes in a human or animal subject. A further object of the invention is a method to determine whether a subject will benefit of different antihypertensive treatments with regard to their effects on the glucose balance and metabolism. Another object of the invention is a method for the selection of subjects for clinical trials testing antidiabetogenic drugs and compounds with effects on the insulin sensitivity.

The present invention concerns a method for detecting a risk of diabetes, especially type 2 diabetes, or a metabolic syndrome in a subject by determining the pattern of alleles encoding a variant α_{2B}-adrenoceptor i.e. to determine if said subject's genotype of the human α_{2B}-adrenoceptor is variant type, comprising the steps of

a) providing a biological sample taken from the subject to be tested,

b) detecting the presence or absence of variant genotype of the human α_{2B}-adrenoceptor gene in the biological sample, the presence of variant genotype indicating an increased risk of diabetes in said subject.

According to the invention, the method allows the determination whether said subject is of said variant genotype or not, a presence of said variant genotype in the biological sample, such as a blood sample or a buccal swab sample, thus indicating an increased risk of the subject to develop diabetes, and/or indicating the subject is in need for treatment, such as α_{2B}-selective or α_{2B}-nonselective antagonist therapy.

Preferably, the present invention concerns a method for detecting a risk of diabetes or a metabolic syndrome in a subject by determining the pattern of alleles encoding a variant α_2-adrenoceptor gene i.e. to determine if said subject's genotype of the α_2-adrenoceptor gene is of the deletion/deletion (D/D) type, comprising the steps of

a) providing a biological sample taken from the subject to be tested,

b) detecting the presence of α_2-adrenoceptor deletion/deletion (D/D) type in the biological sample, the presence of D/D genotype indicating an increased risk of diabetes or a metabolic syndrome in said subject or the subject's need for α_{2B}-selective or α_{2B}-nonselective antagonist therapy for diabetes.

A further object of the invention is a method for treating diabetes in a diabetic or glucose intolerant subject by reducing the sympathetic tone or activity, lowering blood or tissue norepinephrine or epinephrine concentrations and/or antagonizing α_{2B}-adrenoceptors of the human or animal subject.

The present invention is also directed to a kit for detecting a risk of diabetes, especially type 2 diabetes, or a metabolic syndrome in a subject, comprising means for determining the pattern of alleles encoding a variant α_{2B}-adrenoceptor in a biological sample.

In the spirit of the invention, it is also conceivable that any variation in the α_{2B}-adrenoceptor gene that alters the structure or function of the mature α_{2B}-adrenoceptor protein can be used in predicting the risk of diabetes or a metabolic syndrome, choosing a preferable or avoidable antihypertensive medication, or choosing a preferable or avoidable antidiabetic medication, or that said variant gene or materials connected thereto can be utilised as a part of a kit constructed for said uses.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a DNA molecule encoding a variant human α_{2B}-adrenoceptor, said variant α_{2B}-adrenoceptor protein and a method to assess the risk of individuals to develop diabetes or a metabolic syndrome in mammals as well as a method for the targeting treatment for diabetes and selecting subjects for clinical trials.

The word treating shall also be understood to include preventing.

The concept "a deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates" refers to any deletion of 1 to 12 glutamates, amino acids 298–309 (SEQ ID NO: 4), in an acidic stretch of 18 amino acids 294–311 located in the 3rd intracellular loop of the receptor polypeptide irrespective of the specific location of the deletion in said repeat element, or how many glutamates from said repeat element of 12 glutamates are deleted.

The concept "deletion/deletion (D/D) genotype of the human α_{2B}-adrenoceptor", in short "D/D genotype", refers to a genotype of an individual having both α_{2B}-adrenoceptor alleles code for a variant α_{2B}-adrenoceptor with a deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates, amino acids 298–309, in an acidic stretch of 18 amino acids 294–311 (SEQ ID NO: 4), located in the 3rd intracellular loop of the receptor polypeptide. Correspondingly "deletion/insertion (D/I) genotype" refers to a genotype having one of the gene alleles code for an α_{2B}-adrenoceptor with a said deletion and the other without a said deletion, i.e. with a respective insertion, and thus the "insertion/insertion (I/I) genotype" refers to a

genotype having both alleles code for an α_{2B}-adrenoceptor without said deletion or deletions.

The term "metabolic syndrome" is defined in the Experimental Section.

A common variant form (SEQ ID NO: 1) of the human α_{2B}-AR gene (SEQ ID NO: 3) has been identified (Heinonen et al. 1999). This variant gene encodes a receptor protein (SEQ ID NO: 2) with a deletion of 3 glutamates, amino acids 307-309, from a glutamic acid (Glu) repeat element of 12 glutamates, amino acids 298–309, in an acidic stretch of 18 amino acids 294–311 (SEQ ID NO: 4), located in the 3rd intracellular loop of the receptor polypeptide. This variant gene (SEQ ID NO: 1) was associated with decreased basal metabolic rate (BMR) in a group of obese Finnish subjects (Heinonen et al. 1999). Of the 166 obese subjects, 47 (28 %) were homozygous for the long 12 glutamate repeat element (Glu^{12}/Glu^{12}), whereas 90 (54 %) were heterozygous (Glu^{12}/Glu^{9}) and 29 (17 %) were homozygous for the short form (Glu^{9}/Glu^{9}).

The results to be presented below show that in a population sample of Finnish middle-aged men subjects homozygous for the short form (Glu^{9}/Glu^{9}) described above, thus representing a deletion/deletion (D/D) genotype of the α_{2B}-adrenoceptor, have a significantly elevated risk for diabetes. Based on these results and previous publications referred to above it can be postulated that this D/D genotype is related to an impaired capacity to downregulate α_{2B}-adrenoceptor function during sustained receptor activation. Since altered α_{2B}-adrenoceptor function seems to be of relevance in the pathogenesis of diabetes, it could also be of relevance in subjects with the insertion/deletion (I/D) (heterozygous Glu^{12}/Glu^{9}) and insertion/insertion (I/I) (homozygous Glu^{12}/Glu^{12}) genotypes when other risk factors for diabetes are present. Further, since this specific deletion of 3 glutamates from said glutamic acid repeat element of 12 glutamates, amino acids 298–309, in said acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide seems to be of relevance in diabetes then also other deletions, i.e. deletions of at least 1 glutamate, from said glutamic acid repeat element of 12 glutamates, amino acids 298–309, could be of relevance in the pathogenesis of diabetes, because the 3rd intracellular loop of the receptor polypeptide said repeat element is located in seems to have an essential role in

the down-regulation of the α_{2B}-adrenoceptor. Thus individuals with functional mutations in the α_{2B}-adrenoceptor gene have chronically up-regulated α_{2B}-adrenoceptors, leading to the vasoconstriction of peripheral arteries and reduced muscle and pancreatic blood flow.

α_{2B}-adrenoceptors mediate contraction of arteries, and genetic polymorphism present in the α_{2B}-adrenoceptor gene renders some subjects more susceptible to α_{2B}-adrenoceptor mediated vasoconstriction of the peripheral blood flow regulating arteries and arterioles and associated clinical disorders such as diabetes or a metabolic syndrome. These subjects will especially benefit from treatment with an α_{2B}-adrenoceptor antagonist, and will be at increased risk for adverse effects if subtype-nonselective α_2-agonists are administered to them. Therefore, a gene test recognizing subjects with a deletion variant of the α_{2B}-adrenoceptor gene will be useful in diagnostics and patient selection for specific therapeutic procedures and clinical drug testing trials. A gene test recognizing the D/D genotype of the α_{2B}-adrenoceptor is useful in assessing an individual's risk to develop diabetes related to the D/D genotype. The test can be used to set a specific subdiagnosis of diabetes, based on its genetic etiology.

Furthermore, a gene test recognizing the D/D genotype of the α_{2B}-adrenoceptor is useful in selecting drug therapy for patients with diabetes. Such drugs are e.g. a drug modulating, inhibiting or activating the vascular alpha- or beta-adrenargic receptors of the subjects either directly or through central nervous system effects, for example pindolol, propranolol, sotalol, timolol, acebutolol, atenol, betaxolol, bisoprol, esmolol, metoprolol, seliprol, carvedilol, labetalol, clonidine, moxonidine, prazosin, or indapamid, including α-adrenoceptor antagonists (α_{2B}-selective or nonselective).

For instance, as angiotensin II causes an increase of noradrenaline sensitivity, and this effect is at least in part mediated by α-adrenoceptors (Datte et al. 2000), the blood pressure lowering effect of drugs acting through angiotensin II inhibition, such as the angiotensin (AT) receptor blockers, is conceivably enhanced in persons with the D/D genotype of the α_{2B}-adrenoceptor. Such drugs are for example captopril, cinapril, enalapril, imidapril, lisinopril, moexipril, perindopril,

ramipril, trandolapril, candesartan, eprosartan, irbesartan, losartan, valsartan or telmisartan.

A gene test recognizing the D/D genotype of the α_{2B}-adrenoceptor is useful in selecting drug therapy for patients who might be at increased risk for adverse effects of α_2-adrenergic agonists; either it will be possible to avoid the use of α_2-agonists in such patients, or it will be possible to include a specific α_{2B}-antagonist in their therapeutic regimen.

For instance, a nucleic acid sample from a subject can be used for determining if said subject is a carrier of a variant gene. The determination can be carried out either as a DNA analysis according to well known methods, which include direct DNA sequencing of the normal and variant gene, allele specific amplification using the polymerase chain reaction (PCR) enabling detection of either normal or variant sequence, or by indirect detection of the normal or variant gene by various molecular biology methods including e.g. PCR-single stranded conformation polymorphism (SSCP) or conformational analysis (SSCA) method or denaturing gradient gel electrophoresis (DGGE). Determination of the normal or variant gene can also be done by using a restriction fragment length polymorphism (RFLP) method, which is particularly suitable for genotyping large numbers of samples. Similarly, a test based on gene chip or array technology can be easily developed in analogy with many currently existing such tests for single-nucleotide polymorphisms.

The determination can also be carried out at the level of RNA by analyzing RNA expressed at tissue level using various methods. Allele specific probes can be designed for hybridization. Hybridization can be done e.g. using Northern blot, RNase protection assay or in situ hybridization methods. RNA derived from the normal or variant gene can also be analyzed by converting tissue RNA first to cDNA and thereafter amplifying cDNA by an allele specific PCR method.

The presence of variant α_{2B}-adrenoceptor polypeptides indicating the presence of variant gene can be detected by various methods such as hybridization (Western blot), or other antibody based protein assays.

A kit for detecting a risk of diabetes or a metabolic syndrome preferably contains the various components needed for carrying out the diagnostic method according to the present invention. These components are preferably packaged in separate containers and/or vials. The kit may also include instructions for carrying out the method. Thus, for example, some or all of the various reagents and other ingredients needed for carrying out the determination, such as buffers, primers, enzymes, control samples or standards etc. can be packaged separately but provided for use in the same box. Instructions for carrying out the method can be included inside the box, as a separate insert, or as a label on the box and/or on the separate vials. The kit may also contain the necessary computer software needed to interpret the results obtained with the kit, or for utilizing the results from a gene chip used in the method. Preferably, the kit contains a capturing nucleic acid or an antibody specific to a variant α_{2B}-adrenoceptor nucleic acid or polypeptide, respectively.

The publications and other materials used herein to illuminate the background of the invention, and in particular, to provide additional details with respect to its practice, are incorporated herein by reference.

The invention will be described in more detail in the experimental section.

EXPERIMENTAL SECTION

Determination of genomic alleles encoding the α_{2B}-adrenoceptor

DNA fragment analysis of ADRA2B insertion/deletion polymorphism

ADRA2B insertion/deletion mutation

The nucleotide sequence of the primer pair for the amplification of the human ADRA2B gene (alpha2B-adrenergic receptor gene) insertion/deletion polymorphism (SEQ ID NO:3) (SEQ ID NO:1) was as follows 5'– GGG TGT TTG TGG GGC ATC TC –3' (SEQ ID NO:5) and 5'- TGG CAC TGC CTG GGG TTC A -3' (SEQ ID NO:6). A fluorescent label has been added to the 5' end of one of the above mentioned PCR primers. Thus, the pcr fragment is detectable in the capillary electrophoresis conducted with ABI Prism 3100 Genetic Analyzer.

The genomic DNA region of the mutation in question can be amplified with PCR with PTC-220 DNA Dyad PCR machine (MJ Research). The PCR reaction was conducted in a 20 µl volume: the reaction mixture contained 60 ng human genomic DNA (extracted from peripheral blood), 1X PCR Buffer (QIAGEN), 100 µl of each of the nucleotides (dATP, dCTP, dGTP, dTTP), 0.5 µM of each of the primers and 1 unit of the DNA polymerase (QIAGEN, Hot Start Taq DNA polymerase). The PCR conditions need to be determined experimentally, and the following standard protocol can be used as a start: first the reaction was hold 7 minutes at 94°C, then the following three steps were repeated for 35 cycles: 45 secondes at 94°C, 45 secondes at 54°C, 1 minute at 72°C, after which the reaction was kept at 72°C for an additional 5 minutes and finally hold at 4°C.

The insertion/deletion polymorphism of ADRA2B gene concerns an insertion or an deletion of three glutamic acids in the region of 12 Glu aminoacids in the codons 298-309 (SEQ ID NO:3). Thus depending on the genotype, there is either 9 Glu (deletion) (SEQ ID NO:2) or 12 Glu (insertion) (SEQ ID NO:4) at the ADRA2B protein. Depending on whether the amplified allele had an insertion or a deletion in the studied

locus, the size of the PCR product was 91 bp (insertion allele) or 82 bp (deletion allele). Thus, for homozygotes (insertion/insertion or deletion/deletion) only one size of a fragment was detected either 91 bp or 82 bp, respectively. For heterozygotes both of the above mentioned fragments were detected.

Capillary electrophoresis with ABI Prism 3100 Genetic Analyzer

A sample of the ADRA2B insertion/deletion PCR product, 9.00 µl of Hi-Di formamide (Applied Biosystems) and 0.25 µl GeneScan-120 LIZ size standard (Applied Biosystems) were combined in a 96-well 3100 optical microamp plate (Applied Biosystems). The reactions were denatured by placing them at 95°C for 5 minutes and then loaded onto an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Elelctrophoresis data was processed and the genotypes were visualized by using the GeneScan Analysis version 3.7 (Applied Biosystems).

Study Population

The above referred study population of 1426 Finnish middle-aged men subjects including 304 subjects with a specific deletion/deletion (D/D) genotype of the α_{2B}-adrenoceptor is described in more detail in the following:

Knowing the possible involvement of the investigated acidic region in the desensitization mechanism of the receptor we hypothesized that the observed insertion/deletion allelic variation could be associated with insulin resistance, as indicated by serum insulin concentration, the metabolic syndrome and type 2 diabetes. To test this hypothesis, we carried out a population study in middle-aged Finnish men. The study was carried out as part of the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD), which is an ongoing population-based study designed to investigate risk factors for cardiovascular diseases, type 2 diabetes and related outcomes in men from eastern Finland (Salonen 1988). This area is known for its homogenous population (Sajantila et al. 1996) and high coronary morbidity and mortality rates (Keys 1980).

Body-mass index was computed as the ratio of weight (kg) to the square of height (m). Waist circumference was taken as the average of 2 measurements taken after inspiration and after expiration (mean difference between the two measurements ≅ 1.5cm) at the

midpoint between the lowest rib and the iliac crest. Waist-hip ratio was defined as the ratio of waist girth to the circumference of the hips measured at the trochanter major.

Subjects were asked to fast for 12 h before blood sampling. They were also asked to refrain from smoking for 12 h and from consuming alcohol for 3 days before blood draws. Blood glucose was measured at baseline and 11-year follow-up using a glucose dehydrogenase method after precipitation of proteins by trichloroacetic acid. The serum samples for insulin determination were stored at -80°C. Serum insulin was determined with a Novo Biolabs radioimmunoassay kit (Novo Nordisk, Bagsvaerd, Denmark).

Diabetes was defined as fasting blood glucose of 6.7 mmol/L or more or diagnosed diabetes with either dietary, oral or insulin treatment. The metabolic syndrome for men according to the WHO definition was modified for epidemiological studies as proposed by the EGIR (Balkau et al. 1999) and defined as: hyperinsulinemia (fasting insulin levels in the top 25% of the non-diabetic population), impaired fasting glycemia or diabetes and the presence of at least two of the following: abdominal obesity, dyslipidemia (triglycerides ≥1.70 or HDL <0.9 mmol/l), or hypertension (blood pressure ≥140/90 mm Hg or blood pressure medication) (Alberti et al 1998). Insulin resistance was approximated as hyperinsulinemia based on fasting insulin concentrations in the upmost fourth (Balkau et al. 1999). Insulin resistance was also estimated as the bottom fourth of insulin sensitivity as measured by a validated index (QUICKI) based on fasting insulin and glucose concentrations ([log (insulin) + log (glucose)]$^{-1}$)(Katz et al 2000). Hypertension was defined according to the EGIR recommendations at a lower level than the original WHO definition for consistence with current WHO-ISH and Sixth Joint National Committee recommendations (Balkau et al. 1999, The sixth report 1997, Guidelines Subcommittee 1999). Microalbuminuria was not included in the definition (Balkau et al. 1999).

Abdominal obesity was defined according to two definitions: 1) according to the original WHO definition - waist-hip ratio > 0.90 or body-mass index ≥ 30 kg/m^2 (Alberti et al 1998) and 2) modified according to the EGIR recommendation - waist circumference ≥ 94 cm (Balkau et al. 1999).

The metabolic syndrome as defined by the NCEP comprises three or more of the following indications: fasting plasma glucose levels ≥ 6.1 mmol/l (blood glucose levels ≥ 5.6 mmol/l), serum triglycerides ≥ 1.7 mmol/l, serum HDL < 1.0 mmol/l, blood pressure ≥ 135/85 mmHg, waist girth > 102 cm (Executive Summary 2001). Use of waist girth > 94 cm was suggested for men genetically susceptible to insulin resistance (Executive Summary 2001).

Of all the 1426 subjects, 304 (21%) had the homozygous deletion (D/D) genotype. Of 809 subjects who had no diabetes at the baseline examination and for whom 11-year follow-up information was available, 168 were D/D homozygotes. Of the subjects with D/D genotype, 19 (11.3%) developed diabetes during 11 years of follow-up, whereas of the other subjects, 39 (6.1%) developed diabetes. The odds ratio was 2.0 (95% confidence interval 1.1 to 3.5, p=0.028 in 2-sided Fisher's exact test and p=0.021 in a logistic model). Other strongest predictors of diabetes during the follow-up were body-mass index (kg/m2) and the waist-to-hip circumference ratio. In a multivariate logistic model including these covariates, the D/D genotype was associated with a 1.9-fold (95 % confidence interval 1.1 to 3.5, p=0.033) probability (incidence) of diabetes.

The mean fasting baseline serum insulin was 11.1 (mU/L) in 304 D/D homozygotes and 10.5 mU/L in other 1122 genotyped men (p=0.045 for difference in Mann-Whitney U test). The D/D genotype of α_{2B}-adrenoceptor gene was also associated with the prevalence and incidence of metabolic syndrome.

The effects of α-adrenoceptor antagonists, β-adrenoceptor antagonists (beta-blockers) and diuretics on serum insulin and lipids and diabetes was analyzed separately in 278 subjects with an antihypertensive medication, of whom 66 were α_{2B}-AR deletion/deletion homozygotes and 212 had other genotypes. Among the non-homozygotes (the wild I allele carriers), the use of α-adrenoceptor antagonists was associated with a a lowering of both fasting (6.9 vs. 11.3 mU/L, p=0.020) and 2h post glucose load serum insulin concentrations (34.0 vs. 72.3 mU/L, p<0.001), while among the D/D homozygotes, α-adrenoceptor antagonists had no effect on either fasting or post-load serum insulin concentration. Among the α_{2B}-AR deletion homozygotes, the use of β-adrenoceptor antagonist therapy was associated with a lowering of serum total

(5.1 vs 5.8 mmol/L, p=0.010) and LDL cholesterol (3.6 vs. 4.1 mmol/L, p=0.053) concentrations, while among other genotypes, both serum cholesterol and LDL cholesterol tended to be higher among β-blocker users than among non-users. Among the deletion homozygotes, the use of diuretics was associated elevations of serum total (6.0 vs 5.5 mmol/L) and LDL cholesterol (4.4 vs 3.8 mmol/L) and lowering of triglycerides (1.5 vs 2.1 mmol/L, p=0.005), whereas among subjects with other genotypes, serum total and LDL cholesterol tended to be lower and triglycerides higher among the diuretic users.

The prevalence of diabetes (fasting blood glucose of 6.0 mmol/L or more or diagnosed diabetes with either dietary, oral or insulin treatment) at the KIHD 11-year follow-up was in the α_{2B}-AR deletion carriers higher among α-adrenoceptor antagonist users than non-users (25.0% vs 13.2%), while in the non-carriers, the prevalence of diabetes was lower among the users than the non-users (0% vs 12.3%).

Taken together, the known biological properties of the α_{2B}-AR, the homogeneity of the Finnish population, the study design, the relatively large representative study population and the association of diabetes with one trait suggest that the D/D receptor allele is a causal genetic risk factor for diabetes and modifies the effects of α- and β-adrenoceptor modulating drugs and diuretics on glucose and lipid metabolism. The α_{2B}-AR deletion allele homozygocity or carrier status appears to attenuate or abolish the beneficial effect of α-adrenoceptor antagonists on glucose metabolism, to induce cholesterol and LDL cholesterol elevation by diuretics and reverse or attenuate the cholesterol and LDL cholesterol elevating effect of β-blocking agents.

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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CLAIMS

1. A method for detecting a risk of diabetes or a metabolic syndrome in a subject by determining the pattern of alleles encoding a variant α_{2B}-adrenoceptor protein, comprising the steps of

a) providing a biological sample taken from the subject to be tested,

b) detecting the presence or absence of a variant genotype of the human α_{2B}-adrenoceptor in the biological sample, the presence of the variant genotype indicating an increased risk of diabetes or a metabolic syndrome in said subject.

2. The method according to claim 1, where said variant genotype of the human α_{2B}-adrenoceptor gene is any nucleotide variation in the genomic DNA that affects the structure or function of the mature α_{2B}-adrenoceptor protein.

3. The method according to claim 2, wherein said variant genotype of the human α_{2B}-adrenoceptor is a homozygote deletion form of mutation (D/D).

4. The method according to claim 1, wherein the detection step is a DNA-assay.

5. The method according to claim 1, wherein the detection step is carried out using a gene or DNA chip, microarray, strip, panel or similar combination of more than one genes, mutations or RNA expressions to be assayed.

6. The method according to claim 1, wherein the allelic pattern is determined using polymerase chain reaction.

7. The method according to claim 1, wherein the biological sample is any sample that comprises genetic material, preferably a blood sample or buccal swab sample.

8. The method according to claim 1, wherein the detection step is based on a capturing probe, a single strand of cDNA, comprising a nucleotide sequence encoding a variant α_{2B}-adrenoceptor protein or a fragment thereof.

9. The method according to claim 1, wherein the detection step is an analysis of the α_{2B}-adrenoceptor polypeptide or a part thereof.

10. The method according to claim 1, wherein said method is used for determining whether a subject will benefit from a treatment with a drug affecting the norepinephrine sensitivity or sympathetic activity of the subject.

11. The method according to claim 1, wherein said method is used for determining whether a subject will benefit from a treatment with an α- or α_{2B}-adrenoceptor antagonist.

12. The method according to claim 11, wherein said method is used for determining whether the treatment with an α- or α_{2B}-adrenoceptor antagonist will lower or elevate serum insulin concentration.

13. The method according to claim 1, wherein said method is used for determining whether a subject will be at increased risk of hyperglycemia, hyperinsulinemia, a metabolic syndrome and diabetes if a drug modulating, inhibiting or activating the vascular alpha- or beta-adrenargic receptors or a diuretic is administered to the subject.

14. The method according to claim 1, wherein said method is used for determining whether a subject will response to a treatment with diuretics or a drug modulating, inhibiting or activating the vascular alpha- or beta-adrenargic receptors.

15. The method according to claim 1, wherein said method is used for determining whether the treatment with diuretics and a drug modulating, inhibiting or activating the vascular alpha- or beta-adrenargic receptor will lower or elevate serum and plasma cholesterol, LDL cholesterol and triglyceride concentrations.

16. The method according to any one of claims 13 - 15, wherein said drug is pindolol, propranolol, sotalol, timolol, acebutolol, atenol, betaxolol, bisoprol, esmolol, metoprolol, seliprol, carvedilol, labetalol, clonidine, moxonidine, prazosin, or indapamid.

17. The method according to claim 1 further comprising a step of selecting a subject of the D/D genotype for clinical drug trials testing antidiabetic and insulin sensitivity improving effects of compounds.

18. The method according to claim 17, wherein the compound to be tested is a drug affecting the norepinephrine sensitivity or sympathetic activity of the subject.

19. The method according to claim 17, wherein the compound to be tested is a drug modulating, inhibiting or activating the vascular alpha- or beta-adrenergic receptors of the subject either directly or through central nervous system.

20. The method according to claim 1, wherein said diabetes is type 2 diabetes.

21. The method according to claim 1, wherein said metabolic syndrome is defined as hyperinsulinemia (fasting insulin levels in the top 25% of the non-diabetic population), impaired fasting glycemia or diabetes and the presence of at least two of the following: abdominal obesity, dyslipidemia (triglycerides $\geq$1.70 or HDL <0.9 mmol/l), or hypertension (blood pressure $\geq$140/90 mm Hg or blood pressure medication).

22. A method for treating a human or animal subject suffering from type 2 diabetes or for treating vascular complications of diabetes, said method comprising a step of reducing the sympathetic tone, lowering blood or tissue norepinephrine or epinephrine concentrations and/or antagonizing α- or α_{2B}-adrenoceptors of the human or animal subject.

23. The method according to claim 21, wherein said method comprise a step of administering to a subject a compound reducing the sympathetic tone, lowering blood

or tissue norepinephrine or epinephrine concentrations and/or antagonizing α_{2B}-adrenoceptors of the human subject or animal.

24. The method according to claim 23, wherein said compound is a subtype of a selective or nonselective α_{2B}-adrenoceptor antagonist.

25. The method according to claim 23 or 24, wherein said method is a gene therapy or gene transfer method.

26. The method according to claim 25, wherein the gene to be transferred is a non-variant α_{2B}-adrenoceptor gene or a fragment or derivative thereof.

27. A kit for detecting a risk of diabetes or a metabolic syndrome in a subject, comprising means for determining the pattern of alleles encoding a variant α_{2B}-adrenoceptor in a biological sample taken from said subject, and optionally computer software to interpret the results of the determination.

28. The kit according to claim 27 for determining the presence or absence of a variant genotype of the human α_{2B}-adrenoceptor in said biological sample.

29. The kit according to claim 27 or 28 comprising a capturing nucleic acid probe or an antibody.

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gcc aag ggg ggg cct ggg cag ggt gag tcc aag cag ccc cga ccc gac     672
Ala Lys Gly Gly Pro Gly Gln Gly Glu Ser Lys Gln Pro Arg Pro Asp
    210                 215                 220

cat ggt ggg gct ttg gcc tca gcc aaa ctg cca gcc ctg gcc tct gtg     720
His Gly Gly Ala Leu Ala Ser Ala Lys Leu Pro Ala Leu Ala Ser Val
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agt tgg gct gcc ctt ccc aac tca ggc cag ggc cag aag gag ggt gtt      864
Ser Trp Ala Ala Leu Pro Asn Ser Gly Gln Gly Gln Lys Glu Gly Val
        275                 280                 285

tgt ggg gca tct cca gag gat gaa gct gaa gag gag gaa gag gag gag      912
Cys Gly Ala Ser Pro Glu Asp Glu Ala Glu Glu Glu Glu Glu Glu Glu
    290                 295                 300

gag gag gag gaa gag tgt gaa ccc cag gca gtg cca gtg tct ccg gcc      960
Glu Glu Glu Glu Glu Cys Glu Pro Gln Ala Val Pro Val Ser Pro Ala
305                 310                 315                 320

tca gct tgc agc ccc ccg ctg cag cag cca cag ggc tcc cgg gtg ctg     1008
Ser Ala Cys Ser Pro Pro Leu Gln Gln Pro Gln Gly Ser Arg Val Leu
                325                 330                 335

gcc acc cta cgt ggc cag gtg ctc ctg ggc agg ggc gtg ggt gct ata     1056
Ala Thr Leu Arg Gly Gln Val Leu Leu Gly Arg Gly Val Gly Ala Ile
            340                 345                 350

ggt ggg cag tgg tgg cgt cga agg gcg cac gtg acc cgg gag aag cgc     1104
Gly Gly Gln Trp Trp Arg Arg Arg Ala His Val Thr Arg Glu Lys Arg
        355                 360                 365

ttc acc ttc gtg ctg gct gtg gtc att ggc gtt ttt gtg ctc tgc tgg     1152
Phe Thr Phe Val Leu Ala Val Val Ile Gly Val Phe Val Leu Cys Trp
    370                 375                 380

ttc ccc ttc ttc ttc agc tac agc ctg ggc gcc atc tgc ccg aag cac     1200
Phe Pro Phe Phe Phe Ser Tyr Ser Leu Gly Ala Ile Cys Pro Lys His
385                 390                 395                 400

tgc aag gtg ccc cat ggc ctc ttc cag ttc ttc ttc tgg atc ggc tac     1248
Cys Lys Val Pro His Gly Leu Phe Gln Phe Phe Phe Trp Ile Gly Tyr
                405                 410                 415

tgc aac agc tca ctg aac cct gtt atc tac acc atc ttc aac cag gac     1296
Cys Asn Ser Ser Leu Asn Pro Val Ile Tyr Thr Ile Phe Asn Gln Asp
            420                 425                 430

ttc cgc cgt gcc ttc cgg agg atc ctg tgc cgc ccg tgg acc cag acg     1344
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Val Ile Leu Ala Val Leu Thr Ser Arg Ser Leu Arg Ala Pro Gln Asn
35 40 45

Leu Phe Leu Val Ser Leu Ala Ala Ala Asp Ile Leu Val Ala Thr Leu
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Ile Ile Pro Phe Ser Leu Ala Asn Glu Leu Leu Gly Tyr Trp Tyr Phe
65 70 75 80

Arg Arg Thr Trp Cys Glu Val Tyr Leu Ala Leu Asp Val Leu Phe Cys
85 90 95

Thr Ser Ser Ile Val His Leu Cys Ala Ile Ser Leu Asp Arg Tyr Trp
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Ala Val Ser Arg Ala Leu Glu Tyr Asn Ser Lys Arg Thr Pro Arg Arg
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Ile Lys Cys Ile Ile Leu Thr Val Trp Leu Ile Ala Ala Val Ile Ser
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Leu Pro Pro Leu Ile Tyr Lys Gly Asp Gln Gly Pro Gln Pro Arg Gly
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Arg Pro Gln Cys Lys Leu Asn Gln Glu Ala Trp Tyr Ile Leu Ala Ser
165 170 175

Ser Ile Gly Ser Phe Phe Ala Pro Cys Leu Ile Met Ile Leu Val Tyr
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Leu Arg Ile Tyr Leu Ile Ala Lys Arg Ser Asn Arg Arg Gly Pro Arg
195 200 205

Ala Lys Gly Gly Pro Gly Gln Gly Glu Ser Lys Gln Pro Arg Pro Asp
210 215 220

His Gly Gly Ala Leu Ala Ser Ala Lys Leu Pro Ala Leu Ala Ser Val
225 230 235 240

Ala Ser Ala Arg Glu Val Asn Gly His Ser Lys Ser Thr Gly Glu Lys
245 250 255

Glu Glu Gly Glu Thr Pro Glu Asp Thr Gly Thr Arg Ala Leu Pro Pro
260 265 270

Ser Trp Ala Ala Leu Pro Asn Ser Gly Gln Gly Gln Lys Glu Gly Val
275 280 285

Cys Gly Ala Ser Pro Glu Asp Glu Ala Glu Glu Glu Glu Glu Glu Glu
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Glu Glu Glu Glu Glu Cys Glu Pro Gln Ala Val Pro Val Ser Pro Ala
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Ser Ala Cys Ser Pro Pro Leu Gln Gln Pro Gln Gly Ser Arg Val Leu
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Ala Thr Leu Arg Gly Gln Val Leu Leu Gly Arg Gly Val Gly Ala Ile
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Gly Gly Gln Trp Trp Arg Arg Arg Ala His Val Thr Arg Glu Lys Arg
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370 375 380

Phe Pro Phe Phe Phe Ser Tyr Ser Leu Gly Ala Ile Cys Pro Lys His
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Cys Lys Val Pro His Gly Leu Phe Gln Phe Phe Phe Trp Ile Gly Tyr
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Cys Asn Ser Ser Leu Asn Pro Val Ile Tyr Thr Ile Phe Asn Gln Asp
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 2003/000946

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/68 // C07K 14/72
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, EMBASE, CHEM.ABS.DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02066617 A2 (OY JURILAB LTD), 29 August 2002 (29.08.2002), page 9, line 16 - line 25, claim 22	27-29
A		1-21
	--	
A	The Journal of Clinical Endocrinology & Metabolism, Volume 84, 1999, No. 7, Paula Heinonen et al, "Identification of a Three-Amino Acid Deletion in the alpha 2B - Adrenergic Receptor That Is Associated with Reduced Basal Metabolic Rate in Obese Subjects", pages 2429-2433, abstract; page 2432	1-21,27-29
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 2004	19 -02- 2004
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer TERESE PERSSON/BS Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 2003/000946

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing

   ☒ contained in the international application as filed

   ☒ filed together with the international application in computer readable form

   ☐ furnished subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 2003/000946

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	International Journal of Obesity, Volume 25, 2001, K. Sivenius et al, "Effect of a three-amino acid deletion in the alpha2B-adrenergic receptor gene on long-term body weight change in Finnish non-diabetic and type 2 diabetic subjects", pages 1609-1614, abstract --	1-21,27-29
A	US 2002058618 A1 (WURSTER ET AL), 16 May 2002 (16.05.2002), abstract; page 1, column 1, paragraph 4 - column 2, paragraph 3 --	1-21,27-29
A	US 6150389 A (MUNK ET AL), 21 November 2000 (21.11.2000), abstract, table 1 --	1-21,27-29
A	Annu. Rev. Pharmacol. Toxicol., Volume 32, 1993, Robert R. Ruffolo et al, "Pharmacologic and therapeutic applications of alpha2-adrenoceptor subtypes", pages 243-79 --	1-21,27-29
P,A	Clinical Autonomic Research, Volume 13, 2003, Gerasimos P. Sykiotis et al, "The alpha2B adrenergic receptor deletion/insertion polymorphism in morbid obesity", pages 203-207, abstract --	1-21,27-29
P,X	International Journal of Obesity, Volume 27, 2003, Suppl. 1, Siitonen N. et al, "The Association of Polymorphism in the alha2B-Adrenoceptor Gene with Type 2 Diabetes and Obesity", Column T2:P2-017, abstract -- --------	1-9,20

WO	02066617	A2	29/08/2002	EP	1368454 A	10/12/2003
				FI	20010323 A,V	21/08/2002
				US	2003003470 A	02/01/2003
US	2002058618	A1	16/05/2002	US	6521632 B	18/02/2003
				AU	3551001 A	20/08/2001
				BR	0108221 A	05/03/2003
				CA	2399421 A	16/08/2001
				CZ	20022884 A	12/02/2003
				EE	200200435 A	15/12/2003
				EP	1253926 A	06/11/2002
				HU	0300032 A	28/05/2003
				IL	151017 D	00/00/0000
				JP	2003522148 T	22/07/2003
				NO	20023773 A	09/08/2002
				SK	11472002 A	04/02/2003
				WO	0158454 A	16/08/2001
US	6150389	A	21/11/2000	US	6319935 B	20/11/2001
				US	6569884 B	27/05/2003
				US	2002049241 A	25/04/2002
				US	2003187047 A	02/10/2003
				US	5731337 A	24/03/1998
				AU	687584 B	26/02/1998
				AU	3007695 A	09/02/1996
				CA	2194702 A	25/01/1996
				DE	69524223 D,T	22/08/2002
				EP	0770068 A,B	02/05/1997
				ES	2168373 T	16/06/2002
				JP	10502643 T	10/03/1998
				WO	9601813 A	25/01/1996

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 2003/000946

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 22-26
because they relate to subject matter not required to be searched by this Authority, namely:

see extra sheet

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 2003/000946

Box II.1

Claims 22-26 relate to methods of treatment of the human or animal body by surgery or by therapy or diagnostic methods practiced on the human or animal body (PCT Rule 39.1(iv)). No search has been carried out for these claims.